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(54) **METHODS TO CLONE mRNA**

METHODE UM MRNA ZU KLONIEREN

PROCEDES DE CLONAGE D'ARN MESSAGER

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Description

[0001] This invention relates to methods of detecting and cloning of individual mRNAs.

[0002] The activities of genes in cells are reflected in the kinds and quantities of their mRNA and protein species. Gene expression is crucial for processes such as aging, development, differentiation, metabolite production, progression of the cell cycle, and infectious or genetic or other disease states. Identification of the expressed mRNAs will be valuable for the elucidation of their molecular mechanisms, and for applications to the above processes.

[0003] Mammalian cells contain approximately 15,000 different mRNA sequences, however, each mRNA sequence is present at a different frequency within the cell. Generally, mRNAs are expressed at one of three levels. A few "abundant" mRNAs are present at about 10,000 copies per cell, about 3,000-4,000 "intermediate" mRNAs are present at 300-500 copies per cell, and about 11,000 "low-abundance" or "rare" mRNAs are present at approximately 15 copies per cell. The numerous genes that are represented by intermediate and low frequencies of their mRNAs can be cloned by a variety of well established techniques (see for example Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Press, pp. 8.6-8.35).

[0004] If some knowledge of the gene sequence or protein is had, several direct cloning methods are available. However, if the identity of the desired gene is unknown one must be able to select or enrich for the desired gene product in order to identify the "unknown" gene without expending large amounts of time and resources.

[0005] The identification of unknown genes can often involve the use of subtractive or differential hybridization techniques. Subtractive hybridization techniques rely upon the use of very closely related cell populations, such that differences in gene expression will primarily represent the gene(s) of interest. A key element of the subtractive hybridization technique is the construction of a comprehensive complementary-DNA ("cDNA") library.

[0006] The construction of a comprehensive cDNA library is now a fairly routine procedure. PolyA mRNA is prepared from the desired cells and the first strand of the cDNA is synthesized using RNA-dependent DNA polymerase ("reverse transcriptase") and an oligodeoxynucleotide primer of 12 to 18 thymidine residues. The second stand of the cDNA is synthesized by one of several methods, the more efficient of which are commonly known as "replacement synthesis" and "primed synthesis".

[0007] Replacement synthesis involves the use of ribonuclease H ("RNAase H"), which cleaves the phosphodiester backbone of RNA that is in a RNA:DNA hybrid leaving a 3' hydroxyl and a 5' phosphate, to produce nicks and gaps in the mRNA strand, creating a series of RNA primers that are used by *E. coli* DNA polymerase I, or its "Klenow" fragment, to synthesize the second strand of the cDNA. This reaction is very efficient; however, the cDNAs produced most often lack the 5' terminus of the mRNA sequence.

[0008] Primed synthesis to generate the second cDNA strand is a general name for several methods which are more difficult than replacement synthesis yet clone the 5' terminal sequences with high efficiency. In general, after the synthesis of the first cDNA strand, the 3' end of the cDNA strand is extended with terminal transferase, an enzyme which adds a homopolymeric "tail" of deoxynucleotides, most commonly deoxycytidylate. This tail is then hybridized to a primer of oligodeoxyguanidylate or a synthetic fragment of DNA with an deoxyguanidylate tail and the second strand of the cDNA is synthesized using a DNA-dependent DNA polymerase.

[0009] The primed synthesis method is effective, but the method is laborious, and all resultant cDNA clones have a tract of deoxyguanidylate immediately upstream of the mRNA sequence. This deoxyguanidylate tract can interfere with transcription of the DNA *in vitro* or *in vivo* and can interfere with the sequencing of the clones by the Sanger dideoxynucleotide sequencing method.

[0010] Once both cDNA strands have been synthesized, the cDNA library is constructed by cloning the cDNAs into an appropriate plasmid or viral vector. In practice this can be done by directly ligating the blunt ends of the cDNAs into a vector which has been digested by a restriction endonuclease to produce blunt ends. Blunt end ligations are very inefficient, however, and this is not a common method of choice. A generally used method involves adding synthetic linkers or adapters containing restriction endonuclease recognition sequences to the ends of the cDNAs. The cDNAs can then be cloned into the desired vector at a greater efficiency.

[0011] Once a comprehensive cDNA library is constructed from a cell line, desired genes can be identified with the assistance of subtractive hybridization (see for example Sargent T.D., 1987, *Meth. Enzymol.*, Vol. 152, pp. 423-432; Lee *et al.*, 1991, *Proc. Natl. Acad. Sci., USA,* Vol. 88, pp. 2825-2830). A general method for subtractive hybridization is as follows. The complementary strand of the cDNA is synthesized and radiolabelled. This single strand of cDNA can be made from polyA mRNA or from the existing cDNA library. The radiolabelled cDNA is hybridized to a large excess of mRNA from a closely related cell population. After hybridization the cDNA:mRNA hybrids are removed from the solution by chromatography on a hydroxylapatite column. The remaining "subtracted" radiolabelled cDNA can then be used to screen a cDNA or genomic DNA library of the same cell population.

[0012] Subtractive hybridization removes the majority of the genes expressed in both cell populations and thus enriches for genes which are present only in the desired cell population. However, if the expression of a particular mRNA sequence is only a few times more abundant in the desired cell population than the subtractive population it may not

be possible to isolate the gene by subtractive hybridization.

[0013] Proc. Natl. Acad. Scie. USA Vol. 86, pp. 5673-5677, August 1989 Biochemistry discloses one-sided polymerase chain reaction: The amplification of cDNA a rapid technique, based on the polymerase chain reaction (PCR), for the direct targeting, enhancement, and sequencing of previously uncharacterized cDNAs. This method is not limited to previously sequenced transcripts, since it requires only two adjacent or partially overtapping specific primers from only one side of the region to be amplified. These primers can be located anywhere within the message. The specific primers are used in conjunction with nonspecific primers targeted either to the poly(A)$^+$ region of the message or to an enzymatically synthesized d(A) tail.

[0014] Pairwise combinations of specific and general primers allow for the amplification of regions both 3' and 5' to the point of entry into the message. The amplified PCR products can be cloned, sequenced directly by genomic sequencing, or labeled for sequencing by amplifying with a radioactive primer. We illustrate the power of this approach by deriving the cDNA sequences for the skeletal muscle α-tropomyosins of European common frog (*Rana temporaria*) and zebrafish (*Brachydanio rerio*) using only 300 ng of a total poly(A)$^+$ preparation. In these examples, we gained initial entry into the tropomyosin messages by using heterologous primers (to conserved regions) derived from the rat skeletal muscle α-tropomyosin sequence. The frog and zebrafish sequences are used in an analysis of tropomyosin evolution across the vertebrate phylogenetic spectrum. The results underscore the conservative nature of the tropomyosin molecule and support the notion of a constrained heptapeptide unit as the fundamental structural motif of tropomyosin.

[0015] Nucleic Acids Research, Vol. 19, No. 7, discloses efficient double stranded sequencing of cDNA clones containing long poly(A) tails using anchored poly(DT) primers. Sequencing double stranded DNA templates has become a common and efficient procedure (1) for rapidly obtaining sequence data while avoiding preparation of single stranded DNA. Here we report the applicability of this procedure to sequencing cDNA clones containing long stretches of poly (A). Double stranded templates of cDNAs containing long poly(A) tracts are difficult to sequence with vector primers (e.g. universal M13) which anneal downstream of the poly(A) tail. Sequencing with these primers results in a long poly (T) ladder followed by a sequence which is difficult to read (Fig. 1). In an attempt to solve this problem we synthesized three primers which contain $(dT)_{17}$ and either (dA) or (dC) or (dG) at the 3' end. We reasoned that the presence of these three bases at the 3' end would 'anchor' the primers at the upstream end of the poly(A) tail and allow sequencing of the region immediately upstream of the poly(A) region.

[0016] Anchored primers were synthesized on an Applied Biosystems (ABI) 391 DNA synthesizer and used after purification on Oligonucleotide Purification Cartridges (ABI). For sequencing with anchored primers, 5-10 µg of plasmid DNA was denatured in a total volume of 50 µl containing 0.2 M sodium hydroxide and 0.16 mM EDTA by incubation at 65°C for 10 minutes. The three poly(dT) anchored primers (2 pmol of each) were added and the mixture immediately placed on ice. The solution was then neutralized by the addition of 5 µl of 5 M ammonium acetate pH 7.0. The DNA was precipitated by addition of 150 µl of cold 95% ethanol and the pellet washed twice with cold 70% ethanol. The pellet was dried for 5 minutes and then resuspended in 1 × sequencing buffer (1 × = 40 mM Tris-HCl pH 7.5, 20 mM MgCl, 50 mM NaCl). Primers were annealed by heating the solution for 2 minutes at 65°C followed by slow cooling to room temperature. Sequencing reactions, using modified T7 DNA polymerase (Sequenase, United States Biochenucals). were then carried out using [^{32}P]α-dATP (> 1000 Ci/mmole) according to the protocol supplied with the Sequenase kit. Under these conditions over 300 bp of readable sequence could be obtained (Fig. 1). We have applied this approach to several other poly(A)-containing cDNA clones with similar results. Sequencing of the opposite strand of these cDNAs using insert-specific primers verified that the sequences obtained with the anchored primers occurred directly upstream of the poly(A) region (data not shown).

[0017] The ability to directly obtain sequence immediately upstream from the poly(A) tail of cDNAs, as demonstrated here, should be of particular importance to large scale efforts to gene sequence-tagged sites (STSs) (2) from cDNAs (3).

[0018] Nucleic Acids Research, Vol. 19, No. 13 3747 discloses a novel 3' extension technique using random primers in RNA-PCT.

[0019] In order to obtain sequence 3' to a partial ~2 kb titin seqcence of the >21 kb titin mRNA (1) that was too distant from the poly A tail for 3' RACE methodologies (2). RNA-PCR (3, 4) was done using a primer containing a random hexamer at its 3' end. Four µg of rabbit cardiac muscle total RNA (5) in 25 µL were reverse transcribed per BRL's recommendations using 100 ng RT primer (Figure 1) and 200 U MLV reverse transcriptase (BRL). After RNAse H digestion, 10 µL was used for PCR in 100 µL using primers complementary to either known titin sequence (Figure 1, TS 1) or the RT primer (Figure 1, Y primer), and 30 cycles of 93°C-45 sec, 45°C-1.5 min, 72°C-3.0 min. Although defined fragments from 100-1000 bp were observed after the first PCR (Figure 2a) fragments only, <700 bp purified by Geneclean (Bio 101) re-amplified during the second PCR using primers complementary to the RT primer (Figure 1. X primer Containing a Sail site) or the known titin sequence (Figure 1, TS2 containing a NotI site). Lower or higher concentrations of RT primer resulted in no or very small amplification products, respectively. Only the random hexamer part of the RT primer initiated reverse transcription from 6-bp sequences of titin mRNA that had at least 50% G/C content.

[0020] Final amplification products (Figure 2b) were sequenced by dideoxy chain termination methods using Seque-

nase (US Biochemical) after digestion with NotI and SalI restriction enzymes and ligation into pBluescript (Stratagene). Two clones were sequenced because of the possibility of infidelity of the Taq polymerase. After repeating this entire procedure three times using new sets sof titin-specific primers, the titin sequence was extended 1109-bp (EMBL accession no. X59596). The second amplification step could perhaps be omitted or done asymmetrically for sequencing.

[0021] This technique should also be applicable to 5' exiensions of cDNA clones. Perhaps a modification of it could be applied to extensions of known genomic DNA in either direction.

[0022] The Journal of Cell Biology, Vol. 115, No. 4, November 1991, 887-903 discloses an analysis of vertebrate mRNA sequences: intimations of translational control.

[0023] Five structural features in mRNAs have been found to contribute to the fidelity and efficiency of initiation by eukaryotic ribosomes. Scrutiny of vertebrate cDNA sequences in light of these criteria reveals a set of transcripts-encoding oncoproteins, growth factors, transcription factors, and other regulatory proteins-that seem designed to be translated poorly. Thus, throttling at the level of translation may be a critical component of gene regulation in vertebrates. An alternative interpretation is that some (perhaps many) cDNAs with encumbered 5' noncoding sequences represent mRNA precursors, which would imply extensive regulation at a posttranscriptional step that precedes translation.

[0024] We have discovered a method for identifying, isolating and cloning mRNAs as cDNAs using a polymerase amplification method that employs at least two oligodeoxynucleotide primers. In one approach, the first primer contains sequence capable of hybridizing to a site including sequence that is immediately upstream of the first A ribonucleotide of the mRNA's polyA tail and the second primer contains arbitrary sequence. In another approach, the first primer contains sequence capable of hybridizing to a site including the mRNA's polyA signal sequence and the second primer contains arbitrary sequence. In another approach, the first primer contains arbitrary sequence and the second primer contains sequence capable of hybridizing to a site including the mRNA's Kozak sequence. In another approach, the first primer contains a sequence that is substantially complementary to the sequence of a mRNA having a known sequence and the second primer contains arbitrary sequence. In another approach, the first primer contains arbitiacy sequence and the second primer contains sequence that is substantially identical to the sequence of a mRNA having a known sequence. The first primer is used as a primer for reverse transcription of the mRNA and the resultant cDNA is amplified with a polymerase using both the first and second primers as a primer set.

[0025] Using this method with different pairs of the alterable primers, virtually any or all of the mRNAs from any cell type or any stage of the cell cycle, including very low abundance mRNAs, can be identified and isolated. Additionally a comparison of the mRNAs from closely related cells, which may be for example at different stages of development or different stages of the cell cycle, can show which of the mRNAs are constitutively expressed and which are differentially expressed, and their respective frequencies of expression.

[0026] The "first primer" or "first oligodeoxynucleotide" as used herein is defined as being the oligodeoxynucleotide primer that is used for the reverse transcription of the mRNA to make the first cDNA strand, and then is also used for amplification of the cDNA. The first primer can also be referred to as the 3' primer, as this primer will hybridize to the mRNA and will define the 3' end of the first cDNA strand. The "second primer" as used herein is defined as being the oligodeoxynucleotide primer that is used to make the second cDNA strand, and is also used for the amplification of the cDNA. The second primer may also be referred to as the 5' primer, as this primer will hybridize to the first cDNA strand and will define the 5' end of the second cDNA strand.

[0027] The "arbitrary" sequence of an oligodeoxynucleotide primer as used herein is defined as being based upon or subject to individual judgement or discretion. In some instances, the arbitrary sequence can be entirely random or partly random for one or more bases. In other instances the arbitrary sequence can be selected to contain a specific ratio of each deoxynucleotide, for example approximately equal proportions of each deoxynucleotide or predominantly one deoxynucleotide, or to not contain a specific deoxynucleotide. The arbitrary sequence can be selected to contain, or not to contain, a recognition site for specific restriction endonuclease. The arbitrary sequence can be selected to either contain a sequence that is substantially identical (at least 50 homologous) to a mRNA of known sequence or to not contain sequence from a mRNA of known sequence.

[0028] An oligodeoxynuceotide primer can be either "complementary" to a sequence or "substantially identical" to a sequence. As defined herein, a complementary oligodeoxynucleotide primer is a primer that contains a sequence which will hybridize to an mRNA, that is the bases are complementary to each other and a reverse transcriptase will be able to extend the primer to form a cDNA strand of the mRNA. As defined herein, a substantially identical primer is a primer that contains sequence which is the same as the sequence of an mRNA, that is greater than 50% identical, and the primer has the same orientation as an mRNA thus it will not hybridize to, or complement, an mRNA but such a primer can be used to hybridize to the first cDNA strand and can be extended by a polymerase to generate the second cDNA strand. The terms of art "hybridization" or "hybridize", as used herein, are defined to be the base pairing of an oligodeoxynucleotide primer with a mRNA or cDNA strand. The "conditions under which" an oligodeoxynucleotide hybridizes with an mRNA or a cDNA, as used herein, is defined to be temperature and buffer conditions (that are described later) under which the base pairing of the oligodeoxynucleotide primer with either an mRNA or a cDNA occurs and only a few mismatches (one or two) of the base pairing are permissible.

[0029] An oligonucleotide primer can contain a sequence that is known to be a "consensus sequence" of an mRNA of known sequence. As defined herein, a "consensus sequence" is a sequence that has been found in a gene family of proteins having a similar function or similar properties. The use of a primer that includes a consensus sequence may result in the cloning of additional members of a desired gene family.

[0030] The "preferred length" of an oligodeoxynucleotide primer, as used herein, is determined from the desired specificity of annealing and the number of oligodeoxynucleotides having the desired specificity that are required to hybridize to all the mRNAs in a cell. An oligodeoxynucleotide primer of 20 nucleotides is more specific than an oligodeoxynucleotide primer of 10 nucleotides; however, addition of each random nucleotide to an oligodeoxynucleotide primer increases by four the number of oligodeoxynucleotide primers required in order to hybridize to every mRNA in a cell.

[0031] In one aspect, in general, the invention features a method for identifying and isolating mRNAs by priming a preparation of mRNA for reverse transcription with a first oligodeoxynucleotide primer that contains sequence capable of hybridizing to a site including sequence that is immediately upstream of the first A ribonucleotide of the mRNA's polyA tail, and amplifying the cDNA by a polymerase amplification method using the first primer and a second oligodeoxynucleotide primer, for example a primer having arbitrary sequence, as a primer set.

[0032] In preferred embodiments, the first primer contains at least 1 nucleotide at the 3' end of the oligodeoxynucleotide that can hybridize to an mRNA sequence that is immediately upstream of the polyA tail, and contains at least 11 nucleotides at the 5' end that will hybridize to the polyA tail. The entire 3' oligodeoxynucleotide is preferably at least 13 nucleotides in length, and can be up to 20 nucleotides in length.

[0033] Most preferably, the first primer contains 2 nucleotides at the 3' end of the oligodeoxynucleotide that can hybridize to an mRNA sequence that is immediately upstream of the polyA tail. Preferably, the 2 polyA-non-complementary nucleotides are of the sequence VN, where V is deoxyadenylate ("dA"), deoxyguanylate ("dG"), or deoxycytidylate ("dC"), and N, the 3' terminal nucleotide, is dA, dG, dC, or deoxythymidylate ("dT"). Thus the sequence of a preferred first primer is 5'-TTTTTTTTTTTVN [Seq. ID. No. 1]. The use of 2 nucleotides can provide accurate positioning of the first primer at the junction between the mRNA and its polyA tail, as the properly aligned oligodeoxynucleotide: mRNA hybrids are more stable than improperly aligned hybrids, and thus the properly aligned hybrids will form and remain hybridized at higher temperatures. In preferred applications, the mRNA sample will be divided into at least twelve aliquots and one of the 12 possible VN sequences of the first primer will be used in each reaction to prime the reverse transcription of the mRNA. The use of an oligodeoxynucleotide with a single sequence will reduce the number of mRNAs to be analyzed in each sample by binding to a subset of the mRNAs, statistically 1/12th, thus simplifying the identification of the mRNAs in each sample.

[0034] In some embodiments, the 3' end of the first primer can have 1 nucleotide that can hybridize to an mRNA sequence that is immediately upstream of the polyA tail, and 12 nucleotides at the 5' end that will hybridize to the polyA tail, thus the primer will have the sequence 5'-TTTTTTTTTTTTV [Seq. ID. No. 2]. The use of a single non-polyA-complementary deoxynucleotide would decrease the number of oligodeoxynucleotides that are required to identify every mRNA to 3, however, the use of a single nucleotide to position the annealing of primer to the junction of the mRNA sequence and the polyA tail may result in a significant loss of specificity of the annealing and 2 non-polyA-complementary nucleotides are preferred.

[0035] In some embodiments, the 3' end of the first primer can have 3 or more nucleotides that can hybridize to an mRNA sequence that is immediately upstream of the polyA tail. The addition of each nucleotide to the 3' end will further increase the stability of properly aligned hybrids, and the sequence to hybridize to the polyA tail can be decreased by one nucleotide for each additional non-polyA-complementary nucleotide added. The use of such a first primer may not be practical for rapid screening of the mRNAs contained within a given cell line, as the use of a first primer with more than 2 nucleotides that hybridize to the mRNA immediately upstream of the polyA tail significantly increases the number of oligodeoxynucleotides required to identify every mRNA. For instance, the primer 5'-TTTTTTTTTTVNN [Seq. ID. No. 3] would require the use of 48 separate first primers in order to bind to every mRNA, and would significantly increase the number of reactions required to screen the mRNA from a given cell line. The use of oligodeoxynucleotides with a single random nucleotide in one position as a group of four can circumvent the problem of needing to set up 48 separate reactions in order to identify every mRNA. However as the non-polyA-complementary sequence became longer, it would quickly become necessary to increase the number of reactions required to identify every mRNA.

[0036] In preferred embodiments, the second primer is of arbitrary sequence and is at least 9 nucleotides in length. Preferably the second primer is at most 13 nucleotides in length and can be up to 20 nucleotides in length.

[0037] In another aspect, in general, the invention features a method for preparing and isolating mRNAs by priming a preparation of mRNA for reverse transcription with a first primer that contains a sequence capable of hybridizing to the polyadenylation signal sequence and at least 4 nucleotides that are positioned 5', or 3', or both of the polyadenylation signal sequence; this entire first primer is preferably at least 10 nucleotides in length, and can be up to 20 nucleotides in length. In one preferred embodiment the sequence 5'-NNTTTATTNN [Seq. ID. No. 4] can be chosen such that the sequence is 5'-GCMITATTNC [Seq. ID. No. 5], and the four resultant primers are used together in a single reaction for

the priming of the mRNA for reverse transcription. Once the first cDNA strand has been formed by reverse transcription then the first primer can be used with a second primer, for example and arbitrary sequence primer, for the amplification of the cDNA.

[0038] In one aspect, in general, the invention featums a method for identifying and isolating mRNAs by priming a preparation of mRNA for reverse transcription with a first oligodeoxynucleotide primer to generate a first cDNA strand, and priming the preparation of the second cDNA strand with a second primer that contains sequence substantially identical to the Kozak sequence of mRNA, and amplifying the cDNA by a polymerase amplification method using the first and second primers as a primer set.

[0039] In preferred embodiments, the first and second primers are at least 9 deoxynucleotides in length, and are at most 13 nucleotides in length, and can be up to 20 nucleotides in length. Most preferably the first and second primers are 10 deoxynucleotides in length.

[0040] In preferred embodiments the sequence of the first primer is selected at random, or the first primer contains a selected arbitrary sequence, or the first primer contains a restriction endonuclease recognition sequence.

[0041] In preferred embodiments the sequence of the second primer that contains sequence substantially identical to the Kozak sequence of mRNA has the sequence NNNANNATGN [Seq. ID No. 6], or has the sequence NNNANNATGG [Seq. ID No. 7]. Where N is any of the four deoxynucleotides. Preferably, the second primer has the sequence GCCACCATGG [Seq. ID No. 8]. In some embodiments the first primer may further include a restriction endonuclease recognition sequence that is added to either the 5' or 3' end of the primer increasing the length of the primer by at least 5 nucleotides.

[0042] In another aspect, in general, the invention features a method for identifying and isolating mRNAs by priming a preparation of mRNA for reverse transcription with a first oligodeoxynucleotide primer that contains sequence that is substantially complementary to the sequence of a mRNA having a known sequence, and priming the preparation of the second cDNA strand with a second primer and, amplifying the cDNA by a polymerase amplification method using the first and second primers as a primer set.

[0043] In preferred embodiments, the first and second primers are at least 9 deoxynucleotides in length, and are at most 13 nucleotides in length, and can be up to 20 nucleotides in length. Most preferably the first and second primers are 10 deoxynucleotides in length.

[0044] In preferred embodiments the sequence of the first primer further includes a restriction endonuclease sequence, which may be included within the preferred 10 nucleotides of the primer or may be added to either the 3' or 5' end of the primer increasing the length of the oligodeoxynucleotide primer by at least 5 nucleotides.

[0045] In preferred embodiments the sequence of the second primer is selected at random, or the second primer contains a selected arbitrary sequence, or the second primer contains a restriction endonuclease recognition sequence.

[0046] In another aspect, in general, the invention features a method for identifying and isolating mRNAs by priming a preparation of mRNA for reverse transcription with a first oligodeoxynucleotide primer, and priming the preparation of the second cDNA strand with a second primer that contains sequence that is substantially identical to the sequence of a mRNA having a known sequence and, amplifying the cDNA by a polymerase amplification method using the first and second primers as a primer set.

[0047] In preferred embodiments, the first and second primers are at least 9 deoxynucleotides in length, and are at most 13 nucleotides in length, and can be up to 20 nucleotides in length. Most preferably the first and second primers are 10 deoxynucleotides in length.

[0048] In preferred embodiments the sequence of the first primer is selected at random, or the first primer contains a selected arbitrary sequence, or the first primer contains a restriction endonuclease recognition sequence.

[0049] In preferred embodiments the sequence of the second primer having a sequence that is substantially complementary to the sequence of an mRNA having a known sequence further includes a restriction endonuclease sequence, which may be included within the preferred 10 nucleotides of the primer or may be added to either the 3' or 5' end of the primer increasing the length of the oligodeoxynucleotide primer by at least 5 nucleotides.

[0050] In another aspect, in general, the invention features a method for identifying and isolating mRNAs by priming a preparation of mRNA for reverse transcription with a first oligodeoxynucleotide primer that contains sequence that is substantially complementary to the sequence of a mRNA having a known sequence, and priming the preparation of the second cDNA strand with a second primer that contains sequence that is substantially identical to the Kozak sequence of mRNA, and amplifying the cDNA by a polymerase amplification method using the first and second primers as a primer set.

[0051] In preferred embodiments, the first and second primers are at least 9 deoxynucleotides in length, and are at most 13 nucleotides in length, and can be up to 20 nucleotides in length. Most preferably the first and second primers are 10 deoxynucleotides in length.

[0052] In some preferred embodiments of each of the general aspects of the invention, the amplified cDNAs are separated and then the desired cDNAs are reamplified using a polymerase amplification reaction and the first and second oligodeoxynucleotide primers.

[0053] In preferred embodiments of each of the general aspects of the invention, a set of first and second oligodeoxynucleotide primers can be used, consisting of more than one of each primer. In some embodiments more than one of the first primer will be included in the reverse transcription reaction and more than one each of the first and second primers will be included in the amplification reactions. The use of more than one of each primer will increase the number of mRNAs identified in each reaction, and the total number of primers to be used will be determined based upon the desired method of separating the cDNAs such that it remains possible to fully isolate each individual cDNA. In preferred embodiments a few hundred cDNAs can be isolated and identified using denaturing polyacrylamide gel electrophoresis.

[0054] The method according to the invention is a significant advance over current cloning techniques that utilize subtractive hybridization. In one aspect, the method according to the invention enables the genes which are altered in their frequency of expression, as well as of mRNAs which are constitutively and differentially expressed, to be identified by simple visual inspection and isolated. In another aspect the method according to the invention provides specific oligodeoxynucleotide primers for amplification of the desired mRNA as cDNA and makes unnecessary an intermediary step of adding a homopolymeric tail to the first cDNA strand for priming of the second cDNA strand and thereby avoiding any interference from the homopolymeric tail with subsequent analysis of the isolated gene and its product. In another aspect the method according to the invention allows the cloning and sequencing of selected mRNAs, so that the investigator may determine the relative desirability of the gene prior to screening a comprehensive cDNA library for the full length gene product.

Description of the Preferred Embodiments

Drawings

[0055] Fig. 1 is a schematic representation of the method according to the invention.

[0056] Fig. 2 is the sequence of the 3' end of the N1 gene from normal mouse fibroblast cells (A31) [Seq. ID. No. 9].

[0057] Fig. 3 is the Northern blot of the N1 sequence on total cellular RNA from normal and tumorigenic mouse fibroblast cells.

[0058] Fig. 4 is a sequencing gel showing the results of amplification for mRNA prepared from four sources (lanes 14), using the Kozak primer alone, the AP-1 primer alone, the Kozak and AP-1 primers, the Kozak and AP-2 primers, the Kozak and AP-3 primers, the Kozk and AP-4 primers and the Kozak and AP-5 primers. This gel will be more fully described later.

[0059] Fig. 5 is a partial sequence of the 5' end of a clone, K1, that was cloned from the A1-5 cell line that was cultured at the non-permissive temperature and then shifted to the permissive temperature (32.5°C) for 24 h prior to the preparation of the mRNA. The A1-5 cell line is from a primary rat embryo fibroblast cell line that has been doubly transformed with ras and a temperature sensitive mutation of P^{53} ("P^{53ts}").

General Description, Development of the Method

[0060] By way of illustration a description of examples of the method of the invention follows, with a description by way of guidance of how the particular illustrative examples were developed.

[0061] It is important for operation of the method that the length of the oligodeoxynucleotide be appropriate for specific hybridization to mRNA. In order to obtain specific hybridization, whether for conventional cloning methods or PCR, oligodeoxynucleotides are usually chosen to be 20 or more nucleotides in length. The use of long oligodeoxynucleotides in this instance would decrease the number of mRNAs identified during each trial and would greatly increase the number of oligodeoxynucleotides required to identify every mRNA. Recently, it was demonstrated that 9-10 nucleotide primers can be used for DNA polymorphism analysis by PCR (Williams *et al.*, 1991, *Nuc. Acids Res.*, Vol. 18, pp. 6531-6535).

[0062] The plasmid containing the cloned murine thymidine kinase gene ("TK cDNA plasmid") was used as a model template to determine the required lengths of oligodeoxynucleotides for specific hybridization to a mRNA, and for the production of specific PCR products. The oligodeoxynucleotide primer chosen to hybridize internally in the mRNA was varied between 6 and 13 nucleotides in length, and the oligodeoxynucleotide primer chosen to hybridize at the upstream end of the polyA tail was varied between 7 and 14 nucleotides in length. After numerous trials with different sets and lengths of primers, it was determined that the annealing temperature of 42°C is optimal for product specificity and the internally hybridizing oligodeoxynucleotide should be at least 9 nucleotides in length and a oligodeoxynucleotide that is at least 13 nucleotides in length is requirsd to bind to the upstream end of the polyA tail.

[0063] With reference now to Fig. 1, the method according to the invention is depicted schematically. The mRNAs are mixed with the first primer, for example TTTTTTTTTTTVN [Seq. ID. No. 2] (T_{11}VN) **1**, and reverse transcribed **2** to make the first cDNA strand. The cDNA is amplified as follows. The first cDNA strand is added to the second primer and the first primer and the polymerase in the standard buffer with the appropriate concentrations of nucleotides and

the components are heated to 94°C to denature the mRNA:cDNA hybrid **3**, the temperature is reduced to 42°C to allow the second primer to anneal **4**, and then the temperature is increased to 72°C to allow the polymerase to extend the second primer **5**. The cycling of the temperature is then repeated **6**, **7**, **8**, to begin the amplification of the sequences which are hybridized by the first and second primers. The temperature is cycled until the desired number of copies of each sequence have been made.

[0064] As is well known in the art, this amplification method can be accomplished using thermal stable polymerase or a polymerase that is not thermal stable. When a polymerase that is not thermal stable is used, fresh polymerase must be added after the annealing of the primers to the templates at the start of the elongation or extending step, and the extension step must be carried out at a temperature that is permissible for the chosen polymerase.

[0065] The following examples of the method of the invention are presented for illustrative purposes only. As will be appreciated, the method according to the invention can be used for the isolation of polyA mRNA from any source and can be used to isolate genes expressed either differentially or constitutively at any level, from rare to abundant.

Example 1

[0066] Experimentation with the conditions required for accurate and reproducible results by PCR were conducted with the TK cDNA plasmid and a single set of oligodeoxynucleotide primers; the sequence TTTTTTTTTTTCA ("T_{11}CA") [Seq. ID. No. 10] was chosen to hybridize to the upstream end of the polyA tail and the sequence CTTGATTGCC ("Ltk3") [Seq. ID. No. 11] was chosen to hybridize 288 base pairs ("bp") upstream of the polyA tail. The expected fragment size using these two primers is 299 bp.

[0067] PCR was conducted under standard buffer conditions well known in the art with 10 ng TK cDNA plasmid (buffer and polymerase are available from Perkin Elmer-Cetus). The standard conditions were altered in that the primers were used at concentrations of 2.5 μM T_{11}CA [Seq. ID. No.10], 0.5 μM Ltk3 [Seq. ID. No. 11], instead of 1 μM of each primer. The concentration of the nucleotides ("dNTPs") was also varied over a 100 fold range, from the standard 200 μM to 2 μM. The PCR parameters were 40 cycles of a denaturing step for 30 seconds at 94°C, an annealing step for 1 minute at 42°C, and an extension step for 30 seconds at 72°C. Significant amounts of non-specific PCR products were observed when the dNTP concentration was 200 μM, concentrations of dNTPs at or below 20 μM yielded specifically amplified PCR products. The specificity of the PCR products was verified by restriction endonuclease digest of the amplified DNA, which yielded the expected sizes of restriction fragments. In some instances it was found that the use of up to 5 fold more of the first primer than the second primer also functioned to increase the specificity of the product. Lowering the dNTP concentration to 2 μM allowed the labelling of the PCR products to a high specific activity with [α-^{35}S] dATP, 0.5 μM [α-^{35}S] dATP (Sp. Act. 1200 Ci/mmol), which is necessary for distinguishing the PCR products when resolved by high resolution denaturing polyacrylamide gel electrophoresis, in this case a DNA sequencing gel.

Example 2

[0068] The PCR method of amplification with short oligodeoxynucleotide primers was then used to detect a subset of mRNAs in mammalian cells. Total RNAs and mRNAs were prepared from mouse fibroblasts cells which were either growing normally, "cycling", or serum starved, "quiescent". The RNAs and mRNAs were reverse transcribed with T_{11}CA [Seq. ID. No. 10] as the primer. The T_{11}CA primer [Seq. ID. No. 10] was annealed to the mRNA by heating the mRNA and primer together to 65°C and allowing the mixture to gradually cool to 35°C. The reverse transcription reaction was carried out with Moloney murine leukemia virus reverse transcriptase at 35°C. The resultant cDNAs were amplified by PCR in the presence of T_{11}CA [Seq. ID. No. 10] and Ltk3 [Seq. ID. No. 11], as described in Example 1, using 2 μM dNTPs. The use of the T_{11}CA [Seq. ID. No. 10] and Ltk3 [Seq. ID. No. 11] primers allowed the TK mRNA to be used as an internal control for differential expression of a rare mRNA transcript; TK mRNA is present at approximately 30 copies per cell. The DNA sequencing gel revealed 50 to 100 amplified mRNAs in the size range which is optimal for further analysis, between 100 to 500 nucleotides. The patterns of the mRNA species observed in cycling and quiescent cells were very similar as expected, though some differences were apparent. Notably, the TK gene mRNA, which is expressed during G1 and S phase, was found only in the RNA preparations from cycling cells, as expected, thus demonstrating the ability of this method to separate and isolate rare mRNA species such as TK.

Example 3

[0069] The expression of mRNAs in normal and tumorigenic mouse fibroblast cells was also compared using the T_{11}CA [Seq. ID. No. 10] and Ltk3 [Seq. ID. No. 11] primers for the PCR amplification. The mRNA was reverse transcribed using T_{11}CA [Seq. ID. No. 10] as the primer and the resultant cDNA was amplified by PCR using 2 μM dNTPs and the PCR parameters described above. The PCR products were separated on a DNA sequencing gel. The TK mRNA was present at the same level in both the normal and tumorigenic mRNA preparations, as expected, and provided a good

internal control to demonstrate the representation of rare mRNA species. Several other bands were present in one preparation and not in the other, with a few bands present in only the mRNA from normal cells and a few bands present only in the mRNA from the tumorigenic cells; and some bands were expressed to different levels in the normal and tumorigenic cells. Thus, the method according to the invention can be used to identify genes which are normally continuously expressed (constitutive), and differentially expressed, suppressed, or otherwise altered in their level of expression.

Cloning of the mRNA identified in Example 3

[0070] Three cDNAs that are, the TK cDNA, one cDNA expressed only in normal cells ("N1"), and one cDNA expressed only in tumorigenic cells ("T1"), were recovered from the DNA sequencing gel by electroelution, ethanol precipitated to remove the urea and other contaminants, and reamplified by PCR, in two consecutive PCR amplifications of 40 cycles each, with the primers $T_{11}CA$ [Seq. ID. No. 10] and Ltk3 [Seq. ID. No. 11] in the presence of 20 µM dNTPs to achieve optimal yield without compromising the specificity. The reamplified PCR products were confirmed to have the appropriate sizes and primer dependencies as an additional control the reamplified TK cDNA was digested with two separate restriction endonucleases and the digestion products were also confirmed to be of the correct size.

[0071] The reamplified N1 [Seq. ID. No. 9] was cloned with the TA cloning system, Invitrogen Inc., into the plasmid pCR1000 and sequenced. With reference now to Fig. 2, the nucleotide sequence clearly shows the N1 fragment [Seq. ID. No. 9] to be flanked by the underlined Ltk3 primer **15** at the 5' end and the underlined $T_{11}CA$ primer **16** at the 3' end as expected.

[0072] A Northern analysis of total cellular RNA using a radiolabelled N1 probe reconfirmed that the N1 mRNA was only present in the normal mouse fibroblast cells, and not in the tumorigenic mouse fibroblast cells. With reference now to Fig. 3, the probe used to detect the mRNA is labelled to the right of the figure, and the size of the N1 mRNA can be estimated from the 28S and 18S markers depicted to the left of the figure. The N1 mRNA is present at low abundance in both exponentially growing and quiescent normal cells, lanes 1 and 3, and is absent from both exponentially growing or quiescent tumorigenic cells, lanes 2 and 4. As a control, the same Northern blot was reprobed with a radiolabelled probe for 36B4, a gene that is expressed in both normal and tumorigenic cells, to demonstrate that equal amounts of mRNA, lanes 1-4, were present on the Northern blot.

Example 4

[0073] The comparison of the expression of mRNAs in three cell lines, one of which was tested after culturing under two different conditions, was conducted. The cell lines were a primary rat embryo fibroblast cell line ("REF"), the REF cell line that has been doubly transformed with ras and a mutant of P^{53} ("T101-4"), and the REF cell line that has been doubly transformed with ras and a temperature sensitive mutation of P^{53} ("A1-5"). The A1-5 cell line was cultured at the non-permissive temperature of 37°C, and also cultured at 37°C then shifted to the permissive temperature of 32.5°C for 24 h prior to the preparation of the mRNA. The method of the invention was conducted using the primers "Kozak" and one of five arbitrary sequence primers, "AP-1, AP-2, AP-3. AP-4, or AP-5", as the second and first primers, respectively.

[0074] The sequence of the "Kozak" primer was chosen based upon the published consensus sequence for the translation start site consensus sequence of mRNAs (Kozak, 1991, *Jour. Cell Biology,* Vol. 115, pp. 887-903). A degenerate Kozak primer having sequences substantially identical to the translation start site consensus sequence were used simultaneously, these sequences were 5'-GCCRCCATGG [Seq. ID No. 12], in which the R is dA or dG and thus the oligodeoxynucleotide primer has only one of the given nucleotides which results in a mixture of primers.

[0075] The sequence of the five arbitrary primers was a follows: AP-1 had the sequence 5'-AGCCAGCGAA [Seq. ID. No. 13]; AP-2 had the sequence 5'-GACCGCTTGT [Seq. ID. No. 14]; AP-3 had the sequence 5'-AGGTGACCGT [Seq. ID. No. 15]; AP-4 had the sequence 5'-GGTACTCCAC [Seq. ID. No. 16]; and AP-5 had the sequence 5'-GTTGCGATCC [Seq. ID. No. 17]. These arbitrary sequence primers were chosen arbitrarily. In general each arbitrary sequence primer was chosen to have a GC content of 50-70%.

[0076] The mRNA was reverse transcribed using one of the AP primers, as the first primer, and the resultant first cDNA strand was amplified in the presence of both primers, the AP primer and the degenerate Kozak primer, by PCR using 2 µM NTPs and the PCR parameters described above. The PCR products were separated on a DNA sequencing gel. At least 50-100 amplified cDNA bands were present in each of the cell lines tested, and some bands were expressed to different levels in the different cell lines. As a control a reaction was conducted using each arbitrary primer in the absence of the Kozak primer. No cDNA was generated by the arbitrary primer alone, thus demonstrating that both primers were required to amplify an mRNA into a cDNA.

[0077] With reference now to Fig. 4, the primer sets used for each reaction are shown at the top of the Fig. along the line marked Primers. As a control a reaction was conducted using the primers in the absence of mRNA, and using

AP-1 with mRNA in the absence of the Kozak primer. No cDNA was genezated by the primers in the absence of mRNA or by the arbitrary primer alone, thus demonstrating that mRNA is required for amplification and that both primers were required to amplify an mRNA into a cDNA. The cDNA products of the amplification were loaded in the same order across the gel, thus the REF cell line is shown in each of lanes 1, cell line T101-4 is shown in each of lanes 2, cell line A1-5 cultured at 37°C is shown in each of lanes 3, and cell line A1-5 cultured at 32.5°C is shown in each of lanes 4. Each pair of primers resulted in the amplification of a different set of mRNAs from the cell lines. The reactions which were conducted using the Kozak primer and any of primers AP-1, AP-2, AP-4, or AP-5 as a primer set resulted in the amplification of the same cDNA pattern from each of cell lines REF, T101-4, A1-5 cultured at 37°C and A1-5 cultured at 32.5°C. The amplification of mRNA from each cell line and temperature using the Kozak degenerate primer and the AP-3 primer resulted in the finding of one band in particular which was present in the mRNA prepared from the A1-5 cell line when cultured at 32.5°C for 24 h, and not in any of the other mRNA preparations, as can be seen in Fig. 4 designated as K_1. Thus the method according to the invention may be used to identify genes which are differentially expressed in mutant cell lines.

Cloning of the mRNA identified in Example 4

[0078] The cDNA ("K1") that was expressed only in the A1-5 cell line when cultured at 32.5°C was recovered from the DNA sequencing gel and reamplified using the primers Kozak and AP-3 as described above. The reamplified K_1 cDNA was confirmed to have the appropriate size of approximately 450 bp, and was cloned with the TA cloning system, Invitrogen Inc., into the vector pCRII (Invitrogen, Inc.) according to the manufacturers instructions, and sequenced. With reference now to Fig. 5, the nucleotide sequence clearly shows the K_1 clone to be flanked by the underlined Kozak primer **20** at the 5' end and the underlined AP-3 primer **21** at the 3' end as expected. The 5' end of this partial cDNA is identified in Seq. ID No. 18, and the 3' end of this cDNA is identified in Seq. ID No. 19. This partial sequence is an open reading frame, and a search of the gene databases EMBO and Genbank has revealed the translated amino acid sequence from the 3' portion of K_1 to be homologous to the ubiquitin conjugating enzyme family (UBC enzyme). The translated amino acid sequence of the 3' portion of K_1 is 100% identical to a UBC enzyme from *D. melanogaster* and 75 % identical to the UBC-4 enzyme and 79% identical to the UBC-5 enzyme from the yeast *S. saccharomyces*; and 75% identical to the UBC enzyme from *Arabidopsis thaliana*. The K_1 clone may contain the actual 5' end of this gene, otherwise the Kozak primer hybridized just after the 5' end. This result demonstrates that the method according to the invention can be used to clone the 5' coding sequence of a gene

Use

[0079] The method according to the invention can be used to identify, isolate and clone mRNAs from any number of sources. The method provides for the identification of desirable mRNAs by simple visual inspection after separation, and can be used for investigative research, industrial and medical applications.

[0080] For instance, the reamplified cDNAs can be sequenced, or used to screen a DNA library in order to obtain the full length gene. Once the sequence of the cDNA is known, amino acid peptides can be made from the translated protein sequence and used to raise antibodies. These antibodies can be used for further research of the gene product and its function, or can be applied to medical diagnosis and prognosis. The reamplified cDNAs can be cloned into an appropriate vector for further propagation, or cloned into an appropriate expression vector in order to be expressed, either *in vitro* or *in vivo*. The cDNAs which have been cloned into expression vectors can be used in industrial situations for overproduction of the protein product. In other applications the reamplified cDNAs or their respective clones will be used as probes for *in situ* hybridization. Such probes can also be used for the diagnosis or prognosis of disease.

Other Embodiments

[0081] Other embodiments are within the following claims.

[0082] The length of the oligodeoxynucleotide can be varied dependent upon the annealing temperature chosen. In the preferred embodiments the temperature was chosen to be 42°C and the oligonucleotide primers were chosen to be at least 9 nucleotides in length. If the annealing temperature were decreased to 35°C then the oligonucleotide lengths can be decreased to at least 6 nucleotides in length.

[0083] The cDNA could be radiolabelled with radioactive nucleotides other than ^{35}S, such as ^{32}P and ^{33}P. When desired, non-radioactive imaging methods can also be applied to the method according to the invention.

[0084] The amplification of the cDNA could be accomplished by a temperature cycling polymerase chain reaction, as was described, using a heat stable DNA polymerase for the repetitive copying of the cDNA while cycling the temperature for continuous rounds of denaturation, annealing and extension. Or the amplification could be accomplished by an isothermal DNA amplification method (Walker *et al.*, 1992, *Proc. Natl. Acad. Sci.*, Vol. 89, pp. 392-396). The

isothermal amplification method would be adapted to use for amplifying cDNA by including an appropriate restriction endonuclease sequence, one that will be nicked at hemiphosphorothioate recognition sites and whose recognition site can be regenerated during synthesis with $\alpha^{35}S$ labelled dNTPs.

[0085] Proteins having similar function or similar functional domains are often referred to as being part of a gene family. Many such proteins have been cloned and identified to contain consensus sequences which are highly conserved amongst the members of the family. This conservation of sequence can be used to design oligodeoxynucleotide primers for the cloning of new members, or related members, of a family. Using the method of the invention the mRNA from a cell can be reverse transcribed, and a cDNA could be amplified using at least one primer that has a sequence substantially identical to the sequence of a mRNA of known sequence. Consensus sequences for at least the following families and functional domains have been described in the literature: protein tyrosine kinases (Hanks *et al.,* 1991, *Methods on Enzymology,* Vol. 200, pp. 38-81; Wilks, 1991, *Methods in Enzymology,* Vol. 200, pp. 533-546); homeobox genes; zinc-finger DNA binding proteins (Miller *et al,* 1985, *EMBO Jour.,* Vol. 4, pp. 1609-1614); receptor proteins; the signal peptide sequence of secreted proteins; proteins that localize to the nucleus (Guiochon-Mantel *et al.,* 1989, Vol. 57, pp. 1147-1154); serine proteases; inhibitors of serine proteases; cytokines; the SH2 and SH3 domains that have been described in tyrosine kinases and other proteins (Pawson *et al.,* 1992, *Cell,* Vol 71, pp. 359-362); serine/threonine and tyrosine phosphatases (Cohen, 1991, *Methods in Enzymology,* Vol. 201, pp. 398-408); cyclins and cyclin-dependent protein kinases (CDKs) *(see for ex.,* Keyomarsi *et al.,* 1993, *Proc. Natl. Acad. Sci., USA,* Vol. 90, pp. 1112-1116).

[0086] Primers for any consensus sequence can readily be designed based upon the codon usage of the amino acids. The incorporation of degeneracy at one or more sites allows the designing of a primer which will hybridize to a high percentage, greater than 50%, of the mRNAs containing the desired consensus sequence.

[0087] Primers for use in the method according to the invention could be designed based upon the consensus sequence of the zinc finger DNA binding proteins, for example, based upon the amino acid consensus sequence of the proteins PYVC. Useful primers for the cloning of further members of this family can have the following sequences: 5'-GTAYGCNTGT [Seq. ID. No. 20] or 5'-GTAYGCNTGC [Seq. ID. No. 21), in which the Y refers to the deoxynucleotides dT or dC for which the primer is degenerate at this position, and the N refers to inosine ("I"). The base inosine can pair with all of the other bases, and was chosen for this position of the oligodeoxynucleotide as the codon for valine "V" is highly degenerate in this position. The described oligodeoxynucleotide primers as used will be a mixture of 5'-GTATGCITGT and 5'-GTACGCITGT or a mixture of 5'-GTATGCITGC and 5'-GTACGCITGC.

SEQUENCE LISTING

[0088]

(1) GENERAL INFORMATION:

(i) APPLICANT: Liang, Peng
Pardee, Arthur B.
(ii) TITLE OF INVENTION: Identifying, Isolating and Cloning Messenger RNAs
(iii) NUMBER OF SEQUENCES; 21
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(C) CITY: Boston
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(F) ZIP: 02190

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US

(B) FILING DATE: 11-MAR-1993
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: US 07/850,343
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTTTTTTTT TVN 13

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTTTTTTT TTV 13

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTTTTTTTTT VNN 13

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

NNTTTATTNN 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTTTATTNC 10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

NNNANNATGN 10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

NNNANNATGG 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCCACCATGG 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 260 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTTGATTGCC TCCTACAGCA GTTGCAGGCA CCTTTAGCTG TACCATGAAG TTCACAGTCC 60
GGGATTGTGA CCCTAATACT GGAGTTCCAG ATGAAGATGG ATATGATGAT GAATATGTGC 120
TGGAAGATCT TGAGGTAACT GTGTCTGATC ATATTCAGAA GATACTAAAA CCTAACTTCG 180
CTGCTGCCTG GGAAGAGGTG GGAGGAGCAG CTGCGACAGA GCGTCCTCTT CACAGAGGGG 240
TCCTGGGTGA AAAAAAAAAA 260

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTTTTTTTT TCA 13

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTTGATTGCC 10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCCRCCATGG 10

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCCAGCGAA 10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GACCGCTTGT 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGGTGACCGT 10

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGTACTCCAC 10

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTTGCGATCC 10

(2) IMFOKMATIOM FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GCCGCCATGG CTCTGAAGAG AATCCACAAG GACACCCATG AA 42

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTTGCATTTA CAACAAGAAT TTATCATCCA AATATTAACA GTAATGGCAG CATTTGTCTT 60
GATATTCTAC GGTCACCT 78

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTAYGCNTGT 10

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTAYGCNTGC 10

Claims

1. A method for isolating a DNA complementary to a mRNA in a nucleic acid sample comprising the steps of:

a) contacting the sample with a first oligonucleotide primer under conditions in which said first primer hybridizes with any mRNA at a first site having a complementary base sequence;
b) reverse transcribing the mRNA using a reverse transcriptase and said first primer to produce a first DNA strand complementary to at least a portion of the mRNA upstream from said first site;
c) contacting the first DNA strand with a second oligodeoxynucleotide primer under conditions in which said second primer hybridizes with complementary DNA at a second site;
d) extending the second primer using a DNA polymerase to produce a second DNA strand complementary to the first DNA strand downstream from said second site; and
e) amplifying the first and second DNA strands using a polymerase, said first primer and said second primer to form the complementary DNA; wherein:

i) said first primer hybridizes with mRNA at a site that includes a polyA signal sequence; and/or

ii) said first primer hybridizes at a portion of the polyadenosine (polyA) tail of said mRNA and at least one non-polyA nucleotide immediately upstream of said portion; and/or
iii) said first primer hybridizes at a site including a sequence immediately upstream of a first A ribonucleotide of the mRNAs polyA tail; and/or
iv) said second primer with a base sequence of at least 6 nucleotides and containing an arbitrary sequence and a base sequence containing a Kozak sequence.

2. The method of claim 1, wherein said first primer:

a) hybridizes with mRNA that includes at least two nucleotides upstream from and adjacent to the first A ribonucleotide of the polyA tail;
b) includes at least 13 nucleotides;
c) includes a polyA-complementary region comprising at least 11 nucleotides and, upstream from said polyA-complementary region, a non-polyA complementary region comprising at least one nucleotide optionally the non-polyA complementary region comprising at least 2 contiguous nucleotides.

3. The method of claim 2c), wherein said non-polyA-complementary region comprises 3'-NV, wherein V is one of deoxyadenosine, deoxycytidine, or deoxyguanosine, and N is one of deoxyadenosine, deoxycytidine, deoxyguanosine, or deoxythymidine.

4. The method of claim 1a), wherein said first primer comprises at least 6 deoxyribonucleotides.

5. The method of any one of claims 1 to 4, wherein

a) said second primer comprises at least 6 deoxyribonucleotides; or
b) said second primer includes a randomly selected nucleotide sequence; or
c) said first or the second primer includes deoxyadenosine, deoxycytidine, deoxyguanosine, and deoxythymidine; or
d) said first or second primer includes a restriction endonuclease recognition sequence; or
e) said second primer includes a sequence identical to a sequence contained within a mRNA of known sequence; or
f) at least one of said first or second primers comprises a plurality of oligodeoxynucleotides.

6. A method according to claim 1, comprising:

contacting the mRNA with the first primer under conditions in which said first primer hybridizes with mRNA at a site,
reverse transcribing the mRNA using the reverse transcriptase and said first primer, to produce the first DNA strand,
contacting the first DNA strand with the second primer under conditions in which said second primer hybridizes with the first DNA strand at the second site, which includes a Kozak sequence,
extending the second primer using a DNA polymerase to produce a second DNA strand complementary to the first DNA strand downstream from the site of hybridization of said second primer with said first DNA strand, and
amplifying the first and second DNA strands using a DNA polymerase and said first and second primers.

7. The method of claim 6, wherein said first primer includes a sequence substantially identical to a sequence contained within an mRNA of known sequence.

8. The method according to any one of the preceding claims, wherein

a) said first primer comprises at least 9 deoxyribonucleotides, optionally at least 10 deoxyribonucleotides; or
b) said second primer comprises at least 9 deoxyribonucleotides, optionally at least 10 deoxyribonucleotides; or
c) said first primer includes a selected arbitrary sequence of deoxyribonucleotides; or
d) said first primer or said second primer includes a restriction endonuclease recognition sequence; or
e) at least one of said first or second primers comprises a plurality of oligodeoxynucleotides.

9. A method of comparing the presence or level of individual mRNA molecules in two or more nucleic acid samples, comprising the steps of:

a) providing a first nucleic acid sample including mRNA molecules and performing the method of any one of claims 1 to 9 thereupon to produce a first population of amplification products comprising the complementary DNA;
b) providing a second nucleic acid sample including mRNA molecules and performing the method of any one of claims 1 to 9 thereupon to produce a second population of amplification products comprising the complementary DNA;
c) comparing the presence or level of individual amplification products in the first and second populations of amplification products.

10. The method of claim 9, wherein:

a) said first nucleic acid sample comprises mRNAs expressed in a first cell and said second nucleic acid sample consists of mRNAs expressed in a second cell; or
b) said first nucleic acid sample comprises mRNAs expressed in a cell at a first developmental stage and said second nucleic acid sample comprises mRNAs expressed in said cell at a second developmental stage.

11. The method of claim 9 or 10, wherein said first primer includes a polyA-complementary region comprising at least 11 nucleotides and, immediately downstream from said polyA-complementary region, a non-polyA-complementary region comprising at least one nucleotide, and optionally said polyA-complementary region comprises at least 11 contiguous thymidines.

12. The method of claim 11, wherein said first primer comprises at least 13 nucleotides.

13. The method of claim 9, wherein the nucleotide sequence of said first or said second primer contains a restriction endonuclease recognition site, and optionally at least one of said first or second primers comprises a plurality of oligodeoxynucleotides, and further optionally said plurality of oligonucleotides comprises a plurality of oligodeoxynucleotide molecules having the same nucleotide sequence, or individual oligodeoxynucleotide molecules in said plurality of oligodeoxynucleotides have different nucleotide sequences.

14. The method according to any one of claims 9 to 13, further comprising the step of detecting a difference in the presence or level of an individual amplification product in said first population of amplification products as compared with said second population of amplification products.

15. The method according to any one of claims 9 to 14, wherein the amplifying steps each comprise performing a polymerase chain reaction in which the concentration of dNTPs is at or below approximately 20μM, and/or the amplifying steps each comprise performing a polymerase chain reaction in which the concentration of dNTPs is approximately 2μM.

16. The method according to any one of claims 9 to 15, wherein the step of comparing comprises resolving each of said first and second populations of amplification products by gel electrophoresis and comparing the presence or level of bands of particular sizes.

17. The method according to any one of claims 9 to 16, wherein said first cell comprises a tumorigenic cell and said second cell comprises a normal cell.

18. The method according to any one of claims 9 to 17, further comprising a step of cloning individual amplification products from said first or second populations of amplification products.

19. The method according to any one of claims 9 to 18, wherein the second primer that hybridizes to a second site in said first and second samples hybridizes to the second site which includes NNNRNNATGN.

20. The method according to any one of claims 9 to 19, wherein said first primer has a GC content within the range of about 50-70%.

21. The method according to any one of claims 9 to 20, wherein the nucleotide sequence of said first primer includes

a sequence substantially complementary to a consensus sequence found in a gene family.

22. The method according to any one of claims 9 to 21, wherein said first and second sites are separated from one another so that at least some amplification products in said first and second populations of amplification products have a size in the range of approximately 100-500 basepairs.

23. The method according to claim 14, further comprising a step of isolating an individual amplification product whose presence or level differs in said first and second populations of amplification products.

24. The method of claim 23, further comprising a step of cloning said isolated amplification product into a vector.

25. The method of claim 23 or 24, further comprising either a step of screening a nucleic acid library with said isolated amplification product, or a step of determining the nucleotide sequence of at least a portion of said isolated amplification product.

Patentansprüche

1. Ein Verfahren zur Isolierung einer DNA, die zu einer mRNA in einer Nucleinsäure-Probe komplementär ist, umfassend die Schritte:

   a) Inkontaktbringen der Probe mit einem ersten Oligonucleotid-Primer unter Bedingungen, bei denen besagter erster Primer mit beliebiger mRNA an einer ersten Stelle mit einer komplementären Basensequenz hybridisiert;
   b) reverses Transkribieren der mRMA unter Verwendung einer reversen Transkriptase und besagten ersten Primers, um einen ersten DNA-Strang herzustellen, der mindestens zu einem Teil der mRNA stromaufwärts von besagter erster Stelle komplementär ist;
   c) Inkontaktbringen des ersten DNA-Strangs mit einem zweiten Oligodesoxynucleotid-Primer unter Bedingungen, bei denen besagter zweiter Primer mit komplemertärer DNA an einer zweiten Stelle hybridisiert;
   d) Extension des zweiten Primers unter Verwendung einer DNA-Polymerase, um einen zweiten DNA-Strang herzustellen, der zu dem ersten DNA-Strang stromabwärts von besagter zweiter Stelle komplementär ist; und
   e) Amplifizieren des ersten und zweiten DNA-Strangs unter Verwendung einer Polymerase, besagten ersten Primers und besagten zweiten Primers, um die komplementäre DNA zu bilden: worin:

      i) besagter erster Primer mit mRMA an einer Stelle hybridisiert, die eine polyA-Signalsequenz mit einschließt; und/oder
      ii) besagter erster Primer an einen Teil des Polyadenosin (polyA) Schwanzes von besagter mRNA und mindestens an ein nicht-polyA Nucleotid unmittelbar stromaufwärts von besagtem Teil hybridisiert; und/oder
      iii) besagter erster Primer an eine Stelle hybridisiert, einschließlich einer Sequenz unmittelbar stromaufwärts eines ersten A-Ribonucleotids des polyA Schwanzes der mRNA; und/oder
      iv) besagter zweiter Primer mit einer Basensequenz aus mindestens 6 Nucleotiden und enthaltend eine beliebige Sequenz und eine Basensequenz, die eine Kozak-Sequenz enthält.

2. Das Verfahren nach Anspruch 1, worin besagter erster Primer:

   a) mit mRNA hybridisiert, die mindestens zwei Nucleotide stromaufwärts von und angrenzend an das erste A-Ribonucleotid des polyA-Schwanzes mit einschließt;
   b) mindestens 13 Nucleotide umfasst;
   c) eine polyA-komplementäre Region, umfassend mindestens 11 Nucleotide und, stromaufwärts von besagter polyA-komplementärer Region, eine nicht-polyA-komplementäre Region mit einschließt, umfassend mindestens ein Nucleotid gegebenenfalls die nicht-polyAkomplementäre Region, umfassend mindestens 2 benachbarte Nucleotide.

3. Das Verfahren nach Anspruch 2 c), worin besagte nicht-polyA-komplementäre Region 3'-NV umfasst, worin V ein Desoxyadenosin, Desoxycytidin oder Desoxyguanosin ist und N ein Desoxyadenosin, Desoxycytidin, Desoxyguanosin oder Desoxythymidin ist.

4. Das Verfahren nach Anspruch 1a), worin besagter erster Primer mindestens 6 Desoxyribonucleotide umfasst.

5. Das Verfahren nach einem der Ansprüche 1 bis 4, worin

a) besagter zweiter Primer mindestens 6 Desoxyribonucleotide umfasst; oder
b) besagter zweiter Primer eine zufällig ausgewählte Nucleotid-Sequenz mit einschließt; oder
c) besagter erster oder der zweite Primer Desoxyadenosin, Desoxycytidin, Desoxyguanosin und Desoxythymidin mit einschließt; oder
d) besagter erster oder zweiter Primer eine Restriktionsendonuclease-Erkennungssequenz mit einschließt; oder
e) besagter zweiter Primer eine Sequenz mit einschließt, die mit einer Sequenz identisch ist, die in einer mRNA bekannter Sequenz enthalten ist; oder
f) mindestens besagter erster oder zweiter Primer eine Vielzahl an Oligodesoxynucleotiden umfasst.

6. Ein Verfahren nach Anspruch 1, umfassend:

Inkontaktbringen der mRNA mit dem ersten Primer unter Bedingungen, bei denen besagter erster Primer mit mRNA an einer Stelle hybridisiert,
reverses Transkribieren der mRMA unter Verwendung der reversen Transkriptase und besagten ersten Primers, um einen ersten DNA-Strang herzustellen, Inkontaktbringen des ersten DNA-Strangs mit dem zweiten Primer unter Bedingungen, bei denen besagter zweiter Primer mit dem ersten DNA-Strang an der zweiten Stelle hybridisiert, der eine Kozak-Sequenz mit einschließt,
Extension des zweiten Primers unter Verwendung einer DNA-Polymerase, um einen zweiten DNA-Strang herzustellen, der zu dem ersten DNA-Strang stromabwärts von der Stelle, wo besagter zweiter Primer mit besagtem ersten DNA-Strang hybridisiert, komplementär ist, und Amplifizieren des ersten und zweiten DNA-Strangs unter Verwendung einer DNA-Polymerase und besagten ersten und zweiten Primers.

7. Das Verfahren nach Anspruch 6, worin besagter erster Primer eine Sequenz mit einschließt, die im Wesentlichen mit einer Sequenz identisch ist, die in einer mRNA bekannter Sequenz enthalten ist.

8. Das Verfahren gemäß einem der vorausgehenden Ansprüche, worin

a) besagter erster Primer mindestens 9 Desoxyribonucleotide, gegebenenfalls mindestens 10 Desoxyribinucleotide, umfasst; oder
b) besagter zweiter Primer mindestens 9 Desoxyribonucleotide, gegebenenfalls mindestens 10 Desoxyribinucleotide, umfasst; oder
c) besagter erster Primer eine ausgewählte beliebige Sequenz aus Desoxyribonucleotiden mit einschließt; oder
d) besagter erster oder besagter zweiter Primer eine Restriktionsendonuclease-Erkennungssequenz mit einschließt; oder
e) mindestens besagter erster Primer oder zweiter Primer eine Vielzahl an Oligodesoxynucleotiden umfasst.

9. Ein Verfahren zum Vergleich des Vorhandenseins oder Levels individueller mRNA-Moleküle in zwei oder mehr Nucleinsäure-Proben, umfassend die Schritte:

a) Bereitstellen einer ersten mRNA-Moleküle enthaltenden Nucleinsäure-Probe und Durchführung des Verfahrens nach einem der Ansprüche 1 bis 9, um eine erste Population Amplifikationsprodukte herzustellen, die die komplementäre DNA umfassen;
b) Bereitstellen einer zweiten mRNA-Moleküle enthaltenden Nucleinsäure-Probe und Durchführung des Verfahrens nach einem der Ansprüche 1 bis 9, um eine zweite Population Amplifikationsprodukte herzustellen, die die komplementäre DNA umfassen;
c) Vergleich des Vorhandenseins oder Levels individueller Amplifikationsprodukte in der ersten und zweiten Population Amplifikationsprodukte.

10. Das Verfahren nach Anspruch 9, worin:

a) besagte erste Nucleinsäure-Probe mRNAs umfasst, die in einer ersten Zelle exprimiert sind, und besagte zweite Nucleinsäure-Probe aus mRNAs besteht, die in einer zweiten Zelle exprimiert sind; oder
b) besagte erste Nucleinsäure-Probe mRNAs umfasst, die in einer Zelle in einem ersten Entwicklungsstadium exprimiert sind, und besagte zweite Nucleinsäure-Probe mRNAs umfasst, die in besagter Zelle in einem zwei-

ten Entwicklungsstadium exprimiert sind.

11. Das Verfahren nach Anspruch 9 oder 10, worin besagter erster Primer eine polyA-komplementäre Region, umfassend mindestens 11 Nucleotide und, unmittelbar stromabwärts von besagter polyA-komplementärer Region, eine nicht-polyA-komplementäre Region mit einschließt, umfassend mindestens ein Nucleotid, und gegebenenfalls besagte polyA-komplementäre Region mindestens 11 aneinander liegende Thymidine umfasst.

12. Das Verfahren nach Anspruch 11, worin besagter erster Primer mindestens 13 Nucleotide umfasst.

13. Das Verfahren nach Anspruch 9, worin die Nucleotid-Sequenz von besagtem ersten oder besagtem zweiten Primer eine Restriktionsendonuclease-Erkennungsstelle enthält und gegebenenfalls mindestens besagter erster oder besagter zweiter Primer eine Vielzahl an Oligodesoxynucleotiden umfasst, und außerdem gegebenenfalls besagte Vielzahl an Oligonucleotiden eine Vielzahl an Oligodesoxynucleotid-Molekülen mit der gleichen Nucleotid-Sequenz umfasst, oder einzelne Oligodesoxynuoleotid-Moleküle in besagter Vielzahl an Oligodesoxynucleotiden verschiedene Nuclectid-Sequenzen besitzen.

14. Das Verfahren gemäß einem der Ansprüche 9 bis 13, außerdem umfassend den Schritt der Detektion einer Differenz bei Vorhandensein oder Level eines individuellen Amplifikationsprodukts in besagter erster Population Amplifikationsprodukte verglichen mit besagter zweiter Population Amplifikationsprodukte.

15. Das Verfahren gemäß einem der Ansprüche 9 bis 14, worin die Amplifizierungsschritte jeweils eine Durchführung einer Polymerase-Kettenreaktion umfassen, bei der die Konzentration an dNTPs bei oder unter ungefähr 20 µM liegt, und/oder die Amplifizierungsschritte jeweils eine Durchführung einer Polymerase-Kettenreaktion umfassen, bei der die Konzentration an dNTPs ungefähr 2 µM ist.

16. Das Verfahren gemäß einem der Ansprüche 9 bis 15, worin der Vergleichsschritt eine Auftrennung jeweils der ersten und zweiten Population Amplifikationsprodukte mittels Gelelektrophorese und Vergleich des Vorhandenseins oder Levels von Banden bestimmter Größen umfasst.

17. Das Verfahren gemäß einem der Ansprüche 9 bis 16, worin besagte erste Zelle eine Tumoren-bildende Zelle umfasst und besagte zweite Zelle eine gesunde Zelle umfasst.

18. Das Verfahren gemäß einem der Ansprüche 9 bis 17, außerdem umfassend einen Schritt der Klonierung individueller Amplifikationsprodukte von besagter erster oder zweiter Population Amplifikationsprodukte.

19. Das Verfahren gemäß einem der Ansprüche 9 bis 18, worin der zweite Primer, der an eine zweite Stelle in besagter erster und zweiter Probe hybridisiert, an die zweite Stelle, die NNNRNNATGN einschließt, hybridisiert.

20. Das Verfahren gemäß einem der Ansprüche 9 bis 19, worin besagter erster Primer einen GC-Gehalt im Bereich von etwa 50-70% besitzt.

21. Das Verfahren gemäß einem der Ansprüche 9 bis 20, worin die Nucleotid-Sequenz von besagtem ersten Primer eine Sequenz mit einschließt, die zu einer in einer Genfamilie gefundenen Consensus-Sequenz komplementär ist,

22. Das verfahren gemäß einem der Ansprüche 9 bis 21, worin besagte erste und zweite Stelle voneinander getrennt sind, so dass mindestens einige Amplifikationsprodukte in besagter erster und zweiter Population Amplifikationsprodukte eine Größe im Bereich von ungefähr 100-500 Basenpaare besitzen.

23. Das Verfahren, gemäß Anspruch 14, außerdem umfassend einen Schritt der Isolierung eines individuellen Amplifikationsprodukts, dessen Vorhandensein oder Level in besagter erster und zweiter Population Amplifikationsprodukte unterschiedlich ist.

24. Das Verfahren nach Anspruch 23, außerdem umfassend einen Schritt der Klonierung besagten isolierten Amplifikationsprodukts in einen Vektor.

25. Das Verfahren nach Anspruch 23 oder 24, außerdem umfassend entweder einen Schritt der Durchmusterung einer Nucleinsäure-Bibliothek mit besagten isolierten Amplifikationsprodukt oder einen Schritt der Bestimmung der Nucleotid-Sequenz mindestens eines Teils von besagtem isolierten Amplifikationsprodukt.

Revendications

1. Procédé pour isoler un ADN complémentaire d'un ARNm dans un échantillon d'acide nucléique, comprenant les étapes consistant à :

   a) mettre en contact l'échantillon avec une première amorce oligonucléotidique dans des conditions dans lesquelles ladite première amorce s'hybride avec un ARNm quelconque à un premier site possédant une séquence de bases complémentaires ;
   b) effectuer la transcription inverse de l'ARNm en utilisant une transcriptase inverse et ladite première amorce pour produire un premier brin d'ADN complémentaire d'au moins une portion de l'ARNm en amont dudit premier site ;
   c) mettre en contact le premier brin d'ADN avec une seconde amorce oligodésoxynucléotidique dans des conditions dans lesquelles la seconde amorce s'hybride avec un ADN complémentaire à un second site ;
   d) allonger la seconde amorce en utilisant une ADN polymérase pour produire un second brin d'ADN complémentaire du premier brin d'ADN en aval dudit second site ; et
   e) amplifier les premier et second brins d'ADN en utilisant une polymérase, ladite première amorce et ladite seconde amorce, pour former l'ADN complémentaire ; dans lequel :

      i) ladite première amorce s'hybride à l'ARNm à un site qui contient une séquence signal polyA ; et/ou
      ii) ladite première amorce s'hybride à une portion de la queue polyadénosine (polyA) dudit ARNm et au moins un nucléotide non polyA juste en aval de ladite portion ; et/ou
      iii) ladite première amorce s'hybride à un site contenant une séquence immédiatement en aval d'un premier ribonucléotide A de la queue polyA des ARNm ; et/ou
      iv) ladite seconde amorce s'hybride avec une séquence de base d'au moins 6 nucléotides et contenant une séquence arbitraire et une séquence de base contenant une séquence Kozak.

2. Procédé selon la revendication 1, dans lequel ladite première amorce :

   a) s'hybride avec un ARNm qui contient au moins deux nucléotides en amont du premier ribonucléotide A de la queue polyA, et adjacent à ce dernier ;
   b) contient au moins 13 nucléotides ;
   c) contient une région complémentaire de polyA comprenant au moins 11 nucléotides et, en amont de ladite région complémentaire de polyA, une région non complémentaire de polyA comprenant au moins un nucléotide, la région non complémentaire de polyA comprenant éventuellement au moins 2 nucléotides contigus.

3. Procédé selon la revendication 2c), dans lequel ladite région non complémentaire de polyA comprend 3'-NV, où V est un résidu désoxyadénosine, désoxycytidine ou désoxyguanosine, et N est un résidu désoxyadénosine, désoxycytidine, désoxyguanosine ou désoxythymidine.

4. Procédé selon la revendication 1a), dans lequel ladite première amorce comprend au moins 6 désoxyribonucléotides.

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel

   a) ladite seconde amorce comprend au moins 6 désoxyribonucléotides ; ou
   b) ladite seconde amorce contient une séquence nucléotidique choisie au hasard ; ou
   c) ladite première ou seconde amorce contient un résidu désoxyadénosine, désoxycytidine, désoxyguanosine ou désoxythymidine ; ou
   d) ladite première ou seconde amorce contient une séquence de reconnaissance d'une endonucléase de restriction ; ou
   e) ladite seconde amorce contient une séquence identique à une séquence contenue dans un ARNm de séquence connue ; ou
   f) au moins une desdites première ou seconde amorces comprend une pluralité d'oligodésoxynucléotides.

6. Procédé selon la revendication 1, comprenant les étapes consistant à:

   - mettre en contact l'ARNm avec la première amorce dans des conditions dans lesquelles ladite première amorce s'hybride avec l'ARNm à un site ;

- effectuer la transcription inverse de l'ARNm en utilisant la transcriptase inverse et ladite première amorce, pour produire le premier brin d'ADN ;
- mettre en contact le premier brin d'ADN avec la seconde amorce dans des conditions dans lesquelles la seconde amorce s'hybride avec le premier brin d'ADN au second site, ce qui inclut une séquence Kozak ;
- allonger la seconde amorce en utilisant une ADN polymérase pour produire un second brin d'ADN complémentaire du premier brin d'ADN en aval du site d'hybridation de ladite seconde amorce avec ledit premier brin d'ADN ; et
- amplifier les premier et second brins d'ADN en utilisant une ADN polymérase et lesdites première et seconde amorces.

7. Procédé selon la revendication 6, dans lequel ladite première amorce contient une séquence sensiblement identique à une séquence contenue dans un ARNm de séquence connue.

8. Procédé selon l'une quelconque des revendications précédentes, dans lequel

a) ladite première amorce comprend au moins 9 désoxyribonucléotides, éventuellement au moins 10 désoxyribonucléotides ; ou
b) ladite seconde amorce comprend au moins 9 désoxyribonucléotides, éventuellement au moins 10 désoxyribonucléotides ; ou
c) ladite première amorce contient une séquence choisie arbitrairement de désoxyribonucléotides ; ou
d) ladite première amorce ou ladite seconde amorce contient une séquence de reconnaissance d'une endonucléase de restriction ; ou
e) au moins une desdites première ou seconde amorces comprend une pluralité d'oligodésoxynucléotides.

9. Procédé de comparaison de la présence ou du taux de molécules d'ARNm individuelles dans au moins deux échantillons d'acides nucléiques, comprenant les étapes consistant à :

a) se procurer un premier échantillon d'acide nucléique contenant des molécules d'ARNm et le soumettre au procédé selon l'une quelconque des revendications 1 à 9 afin de produire une première population de produits d'amplification comprenant l'ADN complémentaire ;
b) se procurer un second échantillon d'acide nucléique contenant des molécules d'ARNm et le soumettre au procédé selon l'une quelconque des revendications 1 à 9 afin de produire une seconde population de produits d'amplification comprenant l'ADN complémentaire ;
c) comparer la présence ou le taux des produits d'amplification individuels dans les première et seconde populations de produits d'amplification.

10. Procédé selon la revendication 9, dans lequel :

a) ledit premier échantillon d'acide nucléique comprend des ARNm exprimés dans une première cellule et ledit second échantillon d'acide nucléique se compose d'ARNm exprimés dans une seconde cellule ; ou
b) ledit premier échantillon d'acide nucléique comprend des ARNm exprimés dans une cellule à un premier stade de développement et ledit second échantillon d'acide nucléique comprend des ARNm exprimés dans ladite cellule à un second stade de développement.

11. Procédé selon la revendication 9 ou 10, dans lequel ladite première amorce contient une région complémentaire de polyA comprenant au moins 11 nucléotides et, juste en aval de ladite région complémentaire de polyA, une région non complémentaire de polyA comprenant au moins un nucléotide, et éventuellement ladite région complémentaire de polyA comprend au moins 11 résidus thymidine contigus.

12. Procédé selon la revendication 11, dans lequel ladite première amorce comprend au moins 13 nucléotides.

13. Procédé selon la revendication 9, dans lequel la séquence nucléotidique de ladite première ou seconde amorce contient un site de reconnaissance d'une endonucléase de restriction et, éventuellement, au moins une desdites première ou seconde amorces comprend une pluralité d'oligodésoxynucléotides, et éventuellement aussi ladite pluralité d'oligonucléotides comprend une pluralité de molécules d'oligodésoxynucléotide ayant la même séquence nucléotidique, ou les molécules individuelles d'oligodésoxynucléotide dans ladite pluralité d'oligodésoxynucléotides ont différentes séquences nucléotidiques.

14. Procédé selon l'une quelconque des revendications 9 à 13, comprenant en outre l'étape consistant à détecter une différence dans la présence ou le taux d'un produit d'amplification individuel dans ladite première population de produits d'amplification comparée à ladite seconde population de produits d'amplification.

15. Procédé selon l'une quelconque des revendications 9 à 14, dans lequel les étapes d'amplification comprennent chacune le fait de réaliser une réaction en chaîne par polymérase dans laquelle la concentration des dNTP est inférieure ou égale à environ 20µM, et/ou les étapes d'amplification comprennent chacune le fait de réaliser une réaction en chaîne par polymérase dans laquelle la concentration des dNTP est d'environ 2µM.

16. Procédé selon l'une quelconque des revendications 9 à 15, dans lequel l'étape de comparaison comprend la résolution de chacune desdites première et seconde populations de produits d'amplification par électrophorèse sur gel et la comparaison de la présence ou du taux de bandes de tailles particulières.

17. Procédé selon l'une quelconque des revendications 9 à 16, dans lequel ladite première cellule comprend une cellule oncogène et ladite seconde cellule comprend une cellule normale.

18. Procédé selon l'une quelconque des revendications 9 à 17, comprenant en outre une étape de clonage de produits d'amplification individuels à partir desdites première ou seconde populations de produits d'amplification.

19. Procédé selon l'une quelconque des revendications 9 à 18, dans lequel la seconde amorce qui s'hybride à un second site dans lesdits premier et second échantillons s'hybride au second site qui contient NNNRNNATGN.

20. Procédé selon l'une quelconque des revendications 9 à 19, dans lequel ladite première amorce a une teneur en GC dans la gamme d'environ 50-70%.

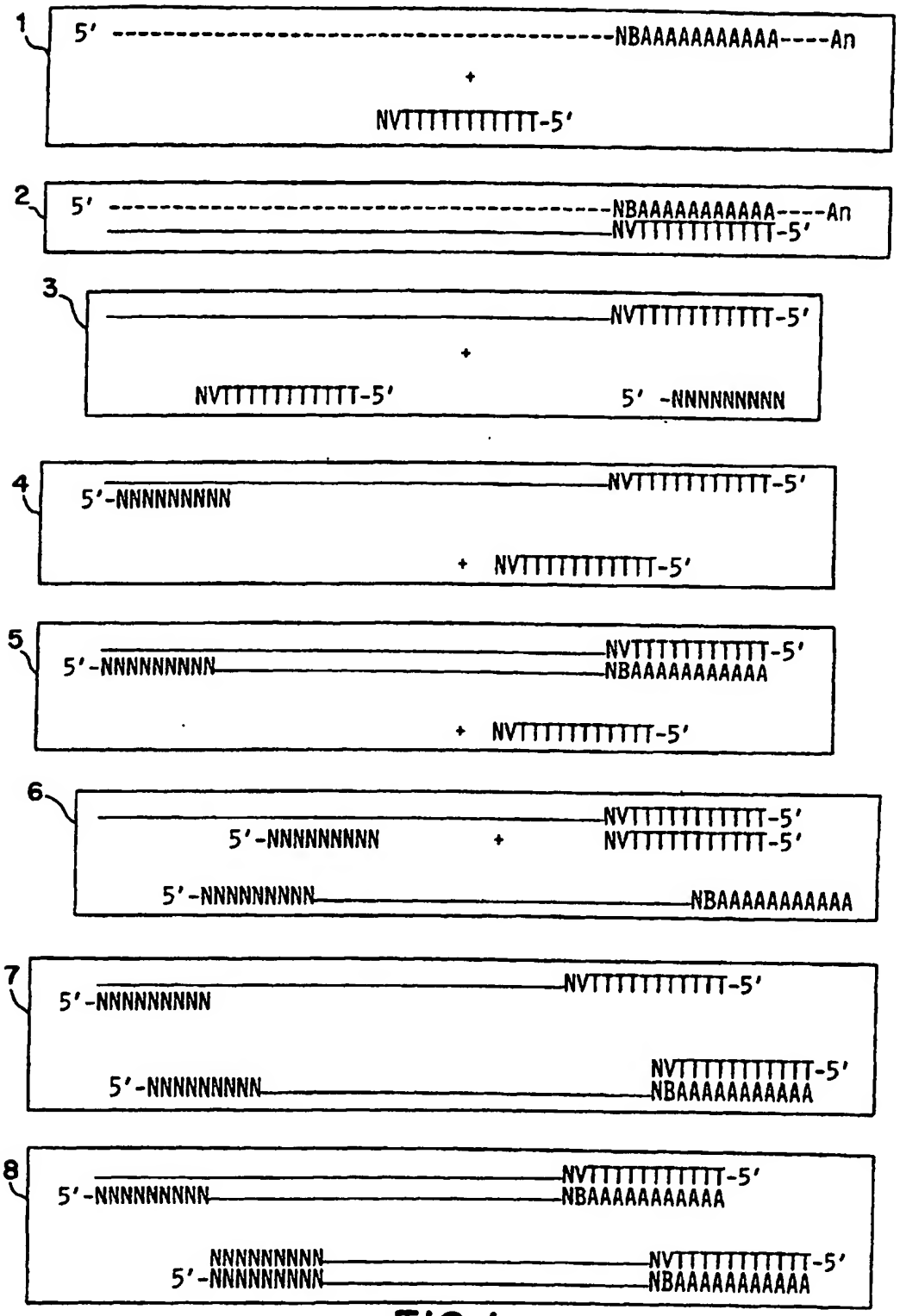
21. Procédé selon l'une quelconque des revendications 9 à 20, dans lequel la séquence nucléotidique de la dite première amorce contient une séquence sensiblement complémentaire d'une séquence consensus trouvée dans une famille de gènes.

22. Procédé selon l'une quelconque des revendications 9 à 21, dans lequel lesdits premier et second sites sont séparés l'un de l'autre de telle sorte qu'au moins certains produits d'amplification dans lesdites première et seconde populations de produits d'amplification aient une taille dans la gamme d'environ 100-500 paires de bases.

23. Procédé selon la revendication 14, comprend en outre une étape d'isolement d'un produit d'amplification individuel dont la présence ou le taux diffère dans lesdites première et seconde populations de produits d'amplification.

24. Procédé selon la revendication 23, comprenant en outre une étape de clonage dudit produit d'amplification isolé dans un vecteur.

25. Procédé selon la revendication 23 ou 24, comprenant en outre soit une étape de criblage d'une banque d'acides nucléiques avec ledit produit d'amplification isolé, soit une étape de détermination de la séquence nucléotidique d'au moins une portion dudit produit d'amplification isolé.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+ NVTTTTTTTTTTT-5'
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FIG. 1

FIG. 2

```
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CTTGATTGCC TCCTACAGCA GTTGCAGGCA CCTTTAGCTG TACCATGAAG TTCACAGTCC
 15
        70         80         90        100        110        120
GGGATTGTGA CCCTAATACT GGAGTTCCAG ATGAAGATGG ATATGATGAT GAATATGTGC
       130        140        150        160        170        180
TGGAAGATCT TGAGGTAACT GTGTCTGATC ATATTCAGAA GATACTAAAA CCTAACTTCG
       190        200        210        220        230        240
CTGCTGCCTG GGAAGAGGTG GGAGGAGCAG CTGCGACAGA GCGTCCTCTT CACAGAGGGG
       250        260
TCCTGGGTGA AAAAAAAAAA
                   16
```

FIG. 3

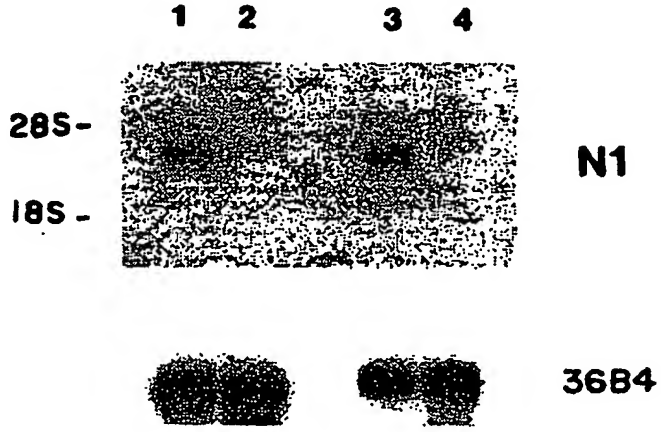
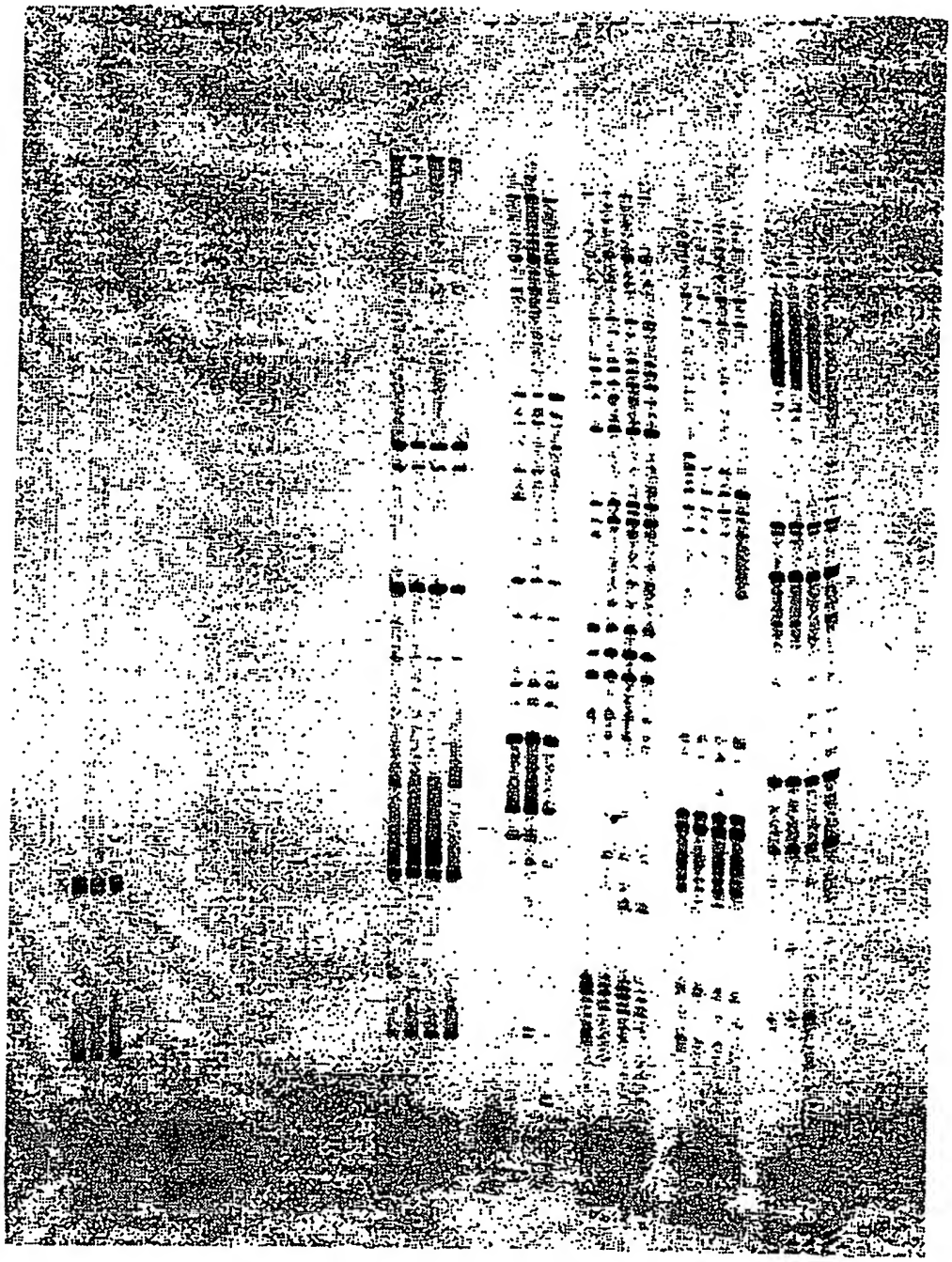


FIG. 4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KOZAK AP-2
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LANE
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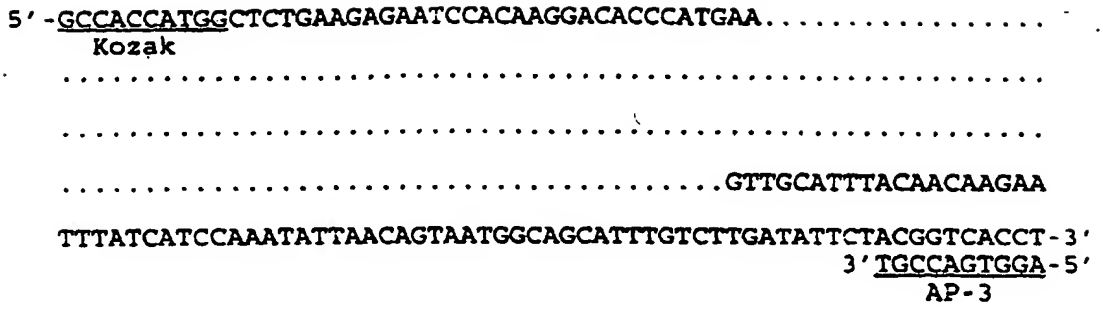
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Kozak
.....
.....
.....GTTGCATTTACAACAAGAA
TTTATCATCCAAATATTAACAGTAATGGCAGCATTTGTCTTGATATTCTACGGTCACCT-3'
3'TGCCAGTGGA-5'
AP-3

FIG. 5